Producing sgRNA-expressing lentivirus for creating chimeras with CHIME

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Method Article

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Abstract

Therapies that target the function of immune cells have significant clinical efficacy in diseases such as cancer and autoimmunity. Although functional genomics has accelerated therapeutic target discovery in cancer, its use in primary immune cells is limited because vector delivery is inefficient and can perturb cell states. Here we describe CHIME: CHimeric IMmune Editing, a CRISPR-Cas9 bone marrow delivery system to rapidly evaluate gene function in innate and adaptive immune cells in vivo without ex vivo manipulation of these mature lineages. This approach enables efficient deletion of genes of interest in major immune lineages without altering their development or function. We use this approach to perform an in vivo pooled genetic screen and identify Ptpn2 as a negative regulator of CD8+ T cell-mediated responses to LCMV Clone 13 viral infection. These findings suggest that this genetic platform can enable rapid target discovery through pooled screening in immune cells in vivo.

Introduction

Current approaches to study genes in immune cells using shRNA or CRISPR technology involve stimulating the immune cells in vitro, transducing them with a lentiviral vector, and transferring them back in vivo. These approaches are limited by inefficient vector delivery, the requirement for stimulation which can perturb cell states, and their use on populations that can be easily transferred in vivo. Here we circumvent those issues by transducing hematopoietic stem cells with a lentiviral gene-targeting sgRNA expression vector, which are then used to make bone marrow chimeras. The resulting immune system of these chimeras is deleted for the gene of interest yet is naive and unstimulated. See figure in Figures section.

Reagents

-DMEM 10% FBS media -Qubit dsDNA assay kit \(Thermo Fisher Scientific Q32853) -SFEM \(STEMCELL Technologies 09600) -45 micron filter \(Whatman EW-29705-54) -SYBR safe DNA gel stain \(Invitrogen S33102) -1 kB DNA ladder \(New England Biolabs N3232L) -IDT sgRNA oligos \(top and bottom) -pXPR_053 lentiviral vector \(Addgene 113591) -BsmBI enyzme \(New England Biolabs R0580L) -Buffer 3.1 \(New England Biolabs B7203S) -1% Agarose gel \(VWR 97064-258) -QIAquick gel extraction kit \(Qiagen 28704) -T4 DNA ligase \(New England Biolabs M0202S) -T4 DNA ligase reaction buffer \(New England Biolabs B0202S) -Stbl3 bacteria \(Invitrogen C737303) -SOC media \(ThermoFisher 15544034) -LB Broth \(Sigma-Aldrich L3022-250G) -LB Broth with Agar \(Sigma-Aldrich L7275-500TAB) -Ampicillin sodium salt \(Sigma-Aldrich A9518-5G) -QIAprep Spin mini prep kit \(Qiagen 27104) -Sequencing primer \(IDT sequence GTGAATAGAGTTAGGCAGGGATATTCACC) -Hispeed Plasmid maxi kit \(Qiagen 12662) -psPAX2 plasmid \(Addgene 12260) -Md2g plasmid \(Addgene 12259) -PEI \(Polysciences 24765-2) -Optimem \(Life Technologies 31985-062) -293x cells \(ATCC CRL-112680) -Polybrene \(Santa Cruz Biotechnology sc-134220) -Mixing beads \(Thermo Fisher Scientific 11-312B) -30 mL syringe \(BD 302832) -Ultracentrifuge tubes \(Beckman Coulter 344058) -T4 PNK enzyme \(New England Biolabs M0201S)

Equipment

-Bacterial shaker -Heat block -Gel electrophoresis kit -Table top centrifuge to accommodate 1.5 mL tubes -Centrifuge to accommodate 50 mL tubes -Ultracentrifuge -SW28 rotor -SW28 rotor tubes -Cell culture incubator -Freezer \(-80°C) -Flow cytometer with violet laser \(BD LSR II for example) -UV Transilluminator -Qubit

Procedure

Day 1: Part 1: Guide Design Step 1: Go to NCBI gene and search for gene of interest Step 2: Find NM sequence for mRNA Step 3: Copy the “NM—“ into http://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design. Select Mouse, 5 output, and don’t show unpicked. Step 4: Click on sgRNA Picking Results to download file. Open text file, Crtl-A, Ctrl-C and paste into new Excel document. Step 5: Pick top 2 guides with best combined score \(incorporates on and off-target effects). Step 6: Copy the sgRNA into “top” and add “G” in front of the sequence Step 7: Go to https://www.idtdna.com/calc/analyzer => Copy top strand => Analyze to generate reverse complementary \(5’ – 3’ direction) Step 8: Add “CACC” to top strand \(5' to 3' orientation) and “AAAC” to bottom complementary strand \(5' to 3' orientation) \(these will form overhands complementary to BsmBI enzyme cut site downstream of the U6 promoter). Step 9: Order these oligos through IDT as lab ready. Part 2: Guide annealing Step 1: Guides come resuspended at concentration of 100 uM. Step 2: Prepare component for annealing reaction. See figure in Figures section. Step 3: Perform the reaction in thermocycler. Sequence: 37°C for 30 minutes, 95°C for 5 minutes, then goes down to 25°C at –5°C/min. Part 3: Digestion of pXPR_053 with BsmBI Step 1: Set up the following digest. See figure in Figures section. Step 2: Place the digest in an Eppendorf tube on a 55°C heat block for 1 hour. Step 3: Prepare a 1% Agarose gel with SYBR safe DNA gel stain. Step 4: Add DNA loading dye to some uncut pXPR_053 and some pXPR_053 from the digestion. Run these 2 samples and a 1 kB DNA marker on the gel. Step 5: Using a UV Transilluminator determine the location of your cut gel, cut out with scalpel, and place in an Eppendorf tube. Step 6: Perform Qiagen gel extraction kit and quantity DNA yield by Qubit. Part 4: Ligation of annealed sgRNAs into digested pXPR_053 Step 1: Set up a ligation reaction, always set up a blank with water instead of sgRNAs. See figure in Figures section. Step 2: Fill up 1L beaker with water. Step 3: Put ligation reaction tubes on floating rack and into the water beaker. Step 4: Put beaker in 4°C cold room and incubate overnight. Day 2: Bacteria transformation Step 1: Thaw Stbl3 bacteria on ice for 10 minutes. Step 2: Place 15 microliter of ligation reaction in the bacteria tube. Finger flick gently to mix. Step 3: Incubate on ice for 30 minutes. Step 4: Heat shock for 45 seconds at 42°C. Step 5: Place back on ice for 5 minutes. Step 6: Add room temp SOC media on top \(1000 microliters). Step 7: Place on shaking heat block \(350 rpm 37 Celsius) for 1 hour. Step 8: Take LB-Amp plates out of fridge to warm to room temp. Label with date and vector + insert name. Step 9: After one hour incubation of bacteria, pellet by placing in table top centrifuge and holding short to spin for 30 seconds. Remove 900 microliters without disturbing pellet and discard. Step 10: Resuspend the pellet in remaining 150 microliters and plate on LB-Amp plate. Step 11: Add 6-8 mixing beads and shake around for 30 seconds. Step 12: Discard beads and let plate sit for 5 minutes to absorb liquid. Step 13: Invert plate and place in

37°C incubator overnight. Day 3: Miniculture Step 1: Prepare LB Broth and 1X Amp. Step 2: Aliquot 3 mL of LB-Amp into mini culture tubes. Step 3: Pick 4 colonies/ligation plate \(except blank plate) using pipette tip, put tips in miniculture tubes. Step 4: Incubate for 8 hours in 37°C shaker incubator. Step 5: Prepare glycerol stocks: 2 drops of 95% glycerol stock and 180 uL of bacteria culture => Freeze at -80°C. Step 6: Perform miniprep using Qiagen Miniprep kit. Quantify DNA using Qubit. Step 7: Submit miniculture for Sanger sequencing with sequencing primer. Step 8: Thaw out some 293x and keep in culture with DMEM 10% FBS media. Day 4: Step 1: Determine which colonies were correct and contain your insert based on sequencing. Step 2: Inoculate a maxi culture with your glycerol stock for 18 hours in 37°C bacterial shaker. Day 5: Step 1: Perform a maxi prep using Qiagen Hispeed Maxi kit. Step 2: Quantify the maxi by Qubit and confirm with Sanger sequencing again. Step 3: Plate 18 million 293x in DMEM 10% FBS into T175s \(for each set of virus you want to make). Day 6: Step 1: Change media on 293x cells, 1 hour before you want to transfect. Step 2: Prepare transfection mix: See figure in Figures section. Step 3: After 1 hour post-media change, drip drop the transfection mixture onto flask. Keep flask level so media can cover all cells. Swirl flask around to mix. Place back in 37°C incubator overnight. Day 7: Step 1: Change media 24h after transfection and let incubate for another 48h before harvesting. Day 9: Step 1: Transfer all media from flask \(take from top corner and do not touch cells) into 50 mL conical. Dispose of the empty flask. Step 2: Spin tubes at 800g for 5 min at 4°C \(to get rid of cells). Step 3: Transfer supernatant to another 50mL conical \(leave behind pellet + 1mL) Step 4: Transfer to 30 mL syringe and filter through 0.45 um filter into ultra-high spin tubes. Then place on ice. Step 5: Clean ultracentrifuge rotors with 70% ethanol and dry out. Step 6: Load ultra-high spin tubes into the rotor. Note the rotor holds 6 tubes so if you have less than 6 samples fill the other tubes with PBS for balance. Step 7: Weigh each tube to ensure difference between each tube is no more than 0.1g. Step 8: Use ultra-centrifuge to spin down supernatant containing lentivirus. We use SW-28 rotor, speed 20,000 rpm, time 2 hours, and temperature 4°C. Step 9: Thaw out SFEM media at room temp meanwhile. Step 10: After centrifugation step, take out tubes and put on ice. Step 11: Invert tubes and dispose of supernatant. Step 12: Dry ultra-spin tube on top of Kim wipe \(keep inverted) for 5 min. Step 13: Add 1 mL of SFEM \(without cytokines) along the side of the tube \(do not disturb bottom). Step 14: Parafilm to cover the top tightly, place tube on rack inside a Styrofoam box. Fill box with ice, make sure virus tube embedded in the ice. Step 15: Put on horizontal shaker in the cold room on super low shaking speed and let shake overnight. Day 10: Step 1: Pre label cryovial to freeze lentivirus. Step 2: Gently resuspend virus in 1mL SFEM \(set pipette at 500 uL), be careful not to create bubbles. Transfer all of volume into labeled cryovial, aliquot out 5 microliter into an Eppendorf, and freeze the rest down in -80°C freezer. Step 3: Plate 293x cells in 96 well flat bottom in DMEM 10% FBS at 25,000 cell per well in 100 microliter. Step 4: Add on top polybrene in 100 microliter DMEM 10% FBS such that polybrene final concentration in the well is 10 ug/mL. Step 5: For each lentivirus plate 2, 0.2, and 0.02 microliter of virus. Make sure to leave a few blank control well. Step 6: Mix wells up and down and place in 37°C tissue culture incubator overnight. Day 11: Step 1: Change media on the 293x-viirus titer plate. Day 13: Step 1: Trypsinize cells to detach, neutralize with media, and plate in a 96 well V bottom plate. Step 2: Read out the Vex+ % \(AmCyan channel), gating on a blank sample, for the different titrations. Step 3: Calculate your lentivirus titer. Calculation is typically done on the dilution of virus that leads to a Vex+% between 1-20%. For example,

assume 0.2 microliter leads to 16% Vex. Equation is: 16% *25000/Volume of virus = Viral titer. So calculating this one: 3200/.2 microliter = 16,000,000 particles/mL.

Timing

13 days total

Troubleshooting

\(1) Low titer-This is commonly due to unhappy 293x cells or improper technique with viral supernatant regarding temperature \(not keeping it cold) and resuspension \(too harsh).

Anticipated Results

Generally for this protocol you should get between 30-100 million viral particles per mL.

Figures

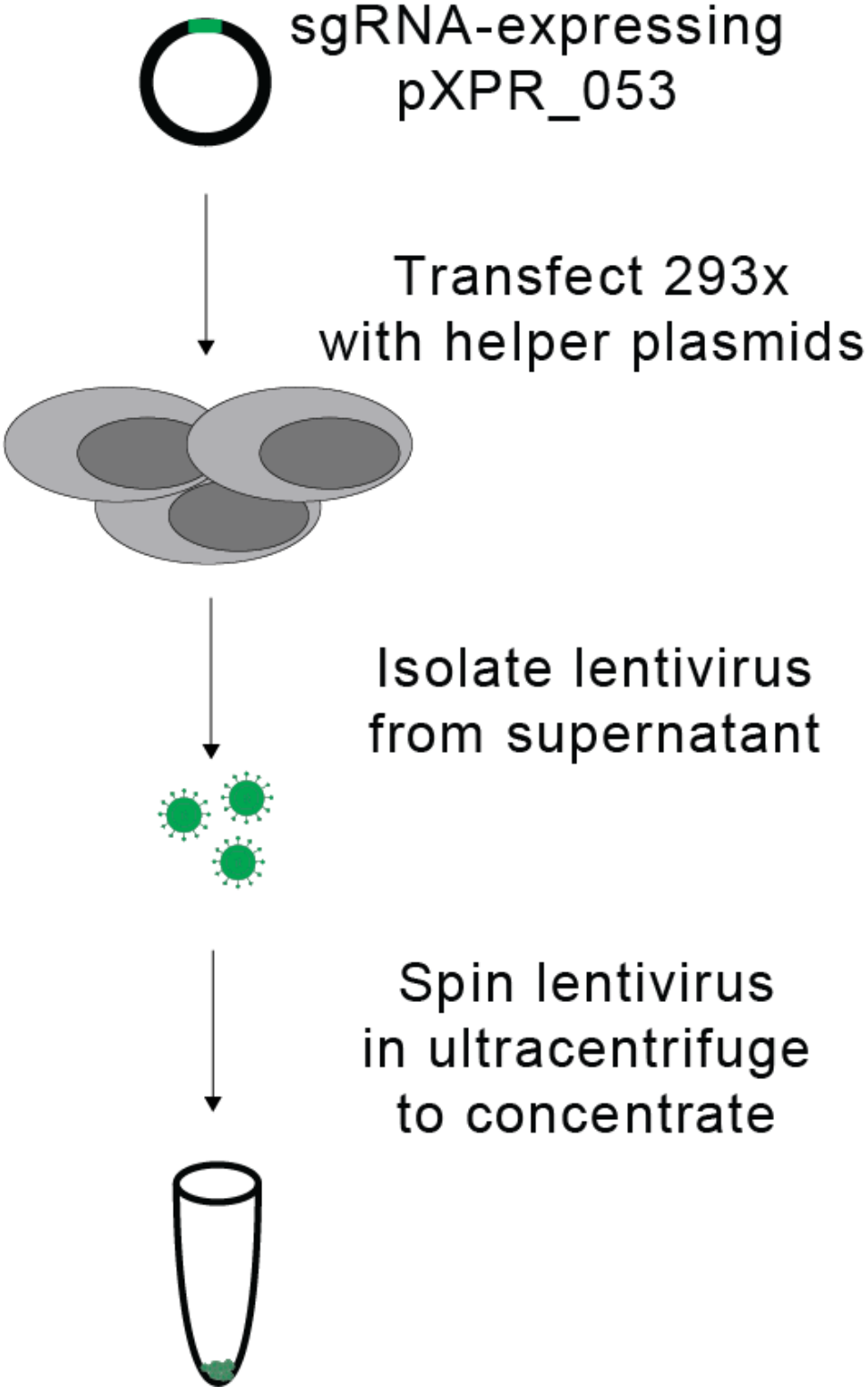

Figure 1

Lentivirus production schematic

Component	Volume (uL)
100 uM sgRNA top	1
100 uM sgRNA bottom	1
T4 ligase buffer (10 mM ATP)	1
T4 PNK	1
MilliQ H2O	6
Total	10

Figure 2

Table 1 Annealing reaction

Component	Volume (uL)
pXPR_053	x (to digest 1 microgram)
Buffer 3.1	2.0
BsmBI	1.0
MilliQ H2O	17-x
Total	20

Figure 3

Table 2 BsmBI digestion reaction

Component	**Volume (uL)**
30ng Digested pXPR_053 (for example 30 ng/uL)	1.0
T4 ligase buffer 10x	2.0
T4 ligase	1.0
Annealed sgRNAs (diluted 1:200 in water)	2.0
MQ H2O	14.0
Total	20.0

Figure 4

Table 3 Ligation reaction

Component	Volume (uL)
Md2g 4.5 ug	x
PsPax2 13.5 ug	y
OptiMem	1800
Vector 18ug	z
PEI (add last!)	108

Figure 5

Table 4 Transfection reaction